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Mechanisms of Adherence of *Candida albicans* to Cultured Human Epidermal Keratinocytes

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We established an in vitro adherence model with primarily cultured human keratinocytes as target cells which allows for the investigation of the molecular mechanisms that are responsible for *Candida albicans* host cell attachment in the initiation of cutaneous candidosis. The extent of *C. albicans* binding to cultured human keratinocytes was dependent on the yeast inoculum size and the incubation temperature. Heat and paraformaldehyde treatment of yeasts completely abolished the binding activity of *C. albicans*. Of the different *Candida* species tested, *C. albicans* was by far the most adhesive species. *C. albicans* adherence was blocked by the acid protease inhibitor pepstatin A and the metabolic inhibitor sodium azide. The latter, however, was much less effective when yeasts were preincubated, suggesting that sodium azide was mainly acting on the keratinocytes. The extracellular matrix protein fibronectin was slightly inhibitory, whereas the fibronectin-derived peptides RGD and RGDS were not able to prevent attachment. PepTite-2000, another RGD-containing synthetic peptide, reduced *C. albicans* adherence by a margin of 25% ($P < 0.005$). CDPGYIGSR-NH_2, which is a synthetic adhesive peptide derived from the laminin B chain, was much more efficient in its inhibitory activity than the RGD peptides and reduced *C. albicans* adherence to cultured human keratinocytes up to 76% ($P < 0.001$). Laminin itself and the synthetic pentapeptide YIGSR were less active. A dose-dependent reduction in adherence was also observed with collagen type III. Additionally, saccharides were tested for their potential to inhibit *C. albicans* attachment to keratinocytes. The most potent competitive saccharide inhibitors of *C. albicans* adherence to human keratinocytes were the amino sugars D-(+)-glucosamine and D-(+)-galactosamine with one isolate of *C. albicans* (4918) and D-(+)-glucosamine and α-D-(+)-fucose with another *C. albicans* isolate (Sp-1). Collectively, our data suggest the existence of multiple molecular mechanisms such as protein-protein, lectin-carbohydrate, and yeast-yeast coaggregational interactions that are responsible for optimal *C. albicans* attachment to cultured human keratinocytes.

The opportunistic yeast *Candida albicans* is a normal saprophyte of the human digestive tract (32). Cutaneous candidosis is a frequently seen infectious disease in early childhood and in adults with predisposing conditions such as diabetes, obesity, cancer, AIDS, and immunosuppressive therapy (6, 29, 32). Typical clinical lesions which sometimes involve large areas of the skin show erythema with many pustular eruptions due to a massive influx of polymorphonuclear granulocytes into the afflicted epidermis (32, 37). Histochemical analysis of skin specimens from *Candida* lesions revealed a confinement of the fungal particles to the epidermal compartment of the skin (37). Under normal circumstances, undamaged skin with an intact stratum corneum, which makes up the outermost part of the epidermis, does not allow for the initiation of candidosis of the skin. Physical damage to the cutaneous stratum corneum, however, enables *C. albicans*, the most frequent *Candida* sp. isolated, to invade and colonize the epidermis (32). Thus, much is known about the prerequisites leading to skin candidosis and the pathophysiological damage caused by *C. albicans* in the skin. However, much less is known about the molecular mechanisms that mediate the initial attachment of *C. albicans* to keratinocytes, the principal target cells within the epidermis.

The fact that *C. albicans* is capable of adhering to plastic surfaces and to cells in various target tissues has widely been accepted as a first step in the pathogenesis of candidosis and can be regarded as an important virulence factor of *C. albicans* compared with other *Candida* spp. (6, 13, 14, 20, 24, 47). In contrast to adherence studies on the candidoses of other tissues in which primarily or continuously cultured cells have been used (10, 26, 27, 39), this does not hold true for cutaneous candidosis. Currently available data are based either on rodent models mimicking human candidosis or on studies done with exfoliated epidermal cells known as corneocytes which have already undergone terminal differentiation (11, 35–37, 45, 46).

In order to gain a better understanding of the molecular mechanisms involved in *C. albicans* adherence to human keratinocytes, we established an adherence model that uses as target cells cultured human keratinocytes derived from neonatal foreskin by the method of Rheinwald and Green (38). This method is characterized by highly reproducible cell culture conditions and yields confluent monolayers of keratinocytes as a substrate for yeast cell attachment. Our results indicate that multiple factors are responsible for optimal mediation of *C. albicans* adherence to cultured human keratinocytes.

MATERIALS AND METHODS

Candida strains and culture conditions. Two strains of *C. albicans* (4918 and Sp-1) were used throughout all experi-

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ments. *C. albicans* 4918 is an original clinical isolate which has been characterized very thoroughly in numerous experimental approaches and was obtained from Richard Calderone, Georgetown University, Washington, D.C. (7, 10, 33, 34). Sp-1 was isolated from a patient with chronic cutaneous candidosis seen in the outpatient unit of the Department of Dermatology at Ludwig-Maximilians-Universität in Munich, Germany. The strain was identified with the API 20C system and was checked for its ability to form germ tubes in the presence of normal human serum (NHS). Other strains used were *C. albicans* 4918-10, a relatively less virulent and less adherent strain derived from its parent wild-type 4918 (34) (also obtained from R. Calderone), *C. guilliermondii* (ATCC 9390), *C. parapsilosis* (ATCC 7330), *C. tropicalis* (ATCC 14526), *C. glabrata* (ATCC 38326), and additional clinical isolates of various *Candida* spp. Strains were maintained on modified Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.). Stocks of each strain were kept frozen at −80°C in Sabouraud medium containing 5% (vol/vol) glycerol. To prepare yeasts for routine adherence assays, *Candida* spp. were grown in yeast nitrogen base (YNB; Difco) for 16 h at 24°C (preliminary experiments at 37°C revealed slightly lower adherence). For some experiments, yeasts were grown in Lee's synthetic medium at 24°C (30) or in YNB with low (50 mM) or high (500 mM) glucose or galactose as the carbohydrate source. Germination of *C. albicans* was induced in Dulbecco's modified Eagle medium (DMEM; ICN Biomedicals, Meckenheim, Germany) for 60 min at 37°C. All liquid cultures were grown in a gyratory shaker at 100 rpm.

Cell culture. Keratinocytes were isolated from human foreskin obtained from routine circumcisions at the Pediatric Surgery Department of Ludwig-Maximilians-Universität essentially as described by Rheinwald and Green (38). The skin was immersed immediately in DMEM containing penicillin (200 IU/ml), gentamicin (200 μg/ml), and amphotericin B (2.5 μg/ml) (all antimicrobial agents were purchased from Sigma, Deisenhofen, Germany) for 2 h and was subsequently dissected into small strips. The skin specimens were further processed by incubation in 0.25% (wt/vol) trypsin in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na-phosphate, 1.5 mM K-phosphate) without Ca^{2+} and Mg^{2+} for 18 h at 4°C. Subsequently, the epidermal layer was peeled off the dermis with a watchmaker forceps and placed in a trypsin-EDTA solution (0.1% [wt/vol] trypsin, 0.02% [wt/vol] EDTA in PBS). Dissociation of the epidermal layer into a single cell suspension was accomplished by gentle aspiration with a Pasteur pipette. The cell suspension was filtered through a sterile gauze and collected in complete fetal calf serum (FCS; ICN Biomedicals). The cells were spun at 300 × *g* for 10 min and resuspended in DMEM-F12 medium (GIBCO-Bethesda Research Laboratories, Berlin, Germany) containing 10% (vol/vol) FCS, cholera toxin (10^{-10} M), epidermal growth factor (10 ng/ml), transferrin (5 μg/ml), and insulin (5 μg/ml) (all reagents obtained from Sigma) without the addition of antibiotic or antimycotic agents. Keratinocytes were plated on mitomycin-treated feeder fibroblasts (derived separately from human foreskin) in 25-cm^2 tissue culture flasks (Falcon; Becton Dickinson Labware, Heidelberg, Germany) at a density of 10^5 cells per cm^2 and were incubated at 37°C with 5% CO_2 at 95% humidity. The medium was changed every other day, and cells were grown to about 90% confluency. Cells were then harvested with trypsin-EDTA solution, spun at 300 × *g*, and resuspended in complete medium without antibiotics and antimycotics. For adherence assays with *Candida* spp., cells were seeded (in the absence of feeder cells) into 96-well microtiter plates (Nunc, Wiesbaden, Germany) at a density of 10^4 cells per well and were grown to confluency in complete DMEM-F12 medium as described above. Under these high Ca^{2+} (2 mM) growth conditions, cultured keratinocytes express involucrin, an early marker of terminal differentiation, which is known to be absent in the basal layer of the skin epithelium (49).

Adherence assay. The keratinocyte monolayer adherence assay as described by Hazen (19) for human intestinal and HeLa cells was used, with some modifications. For routine adherence assays, yeasts were grown in YNB containing 1% (wt/vol) glucose at 24°C for 16 h at 100 rpm. Subsequently, yeasts were washed twice in cold PBS and were adjusted to a stock solution of 5 × 10^6 cells per ml by direct counting of the cell concentration in a hemacytometer. At this point, the yeast suspension was vortexed gently several times to avoid yeast aggregation. The actual inoculum size and yeast viability (CFU) were routinely checked by determining the titer of the yeast suspension on Sabouraud dextrose agar plates. The plates were subsequently incubated at 37°C for 24 h, and colonies were enumerated. Human keratinocyte monolayers prepared and cultured in 96-well microtiter plates as described above were washed twice in plain DMEM at 37°C before they were used in attachment assays. Keratinocyte viability as determined by trypan blue dye exclusion always exceeded 90%. A total cell number of 5 × 10^4 viable yeasts per well was added in a volume of 100 μl of PBS. Incubation was performed for 60 min at 37°C without agitation. Subsequently, each well was gently washed three times with warm PBS to remove nonadherent yeasts. Finally, the keratinocyte monolayers with attached *Candida* organisms were fixed by adding 100 μl of 3.7% (vol/vol) paraformaldehyde in PBS. Quantitation of *Candida* adherence was performed by light microscopy at a ×25 magnification with an inverted microscope (Leitz, Oberkochen, Germany). Each well was counted independently at four random locations (each scaled square counted consisted of an area of 0.0859 mm^2, which equals 11.64^{-1} of a square millimeter) by two individuals. The attachment index per square millimeter was calculated as follows: attachment index (yeasts/mm^2) = (total yeast number counted/4) × 11.64.

In preliminary experiments, light microscopic counting of bound yeasts as described above was evaluated in comparison to enumeration of the CFU of monolayer-attached yeasts. For this purpose, keratinocytes with adhering yeasts were detached by using trypsin-EDTA solution and then two washing steps with PBS. The resulting yeast-keratinocyte suspension was gently sonicated as described before (39), and the titer was determined on Sabouraud dextrose agar plates with subsequent incubation at 37°C for 24 to 36 h. *Candida* colonies were enumerated in quadruplicate samples. More than 95% of the cells originally inoculated onto the monolayer were recoverable and gave rise to colonies. The results obtained by counting the CFU correlated in a statistically significant way with the results obtained by light microscopic evaluation (correlation coefficient [r] = 0.97).

Factors influencing adherence. Various inhibitory agents were tested for their potential to act as antagonists in *C. albicans* adhesion to human keratinocytes. For this purpose, adherence assays were performed as described above except that 5 × 10^5 *C. albicans* yeasts were exposed to the individual inhibitors prior to the adherence assay in a total volume of 100 μl (30 min, 0°C; in PBS) at the concentrations indicated. In some experiments, inhibitory agents were added to the keratinocyte monolayer before adherence assays were performed. All blocking experiments included controls in cell-free, protein-coated wells to rule out coaggregation as the reason for a change in the attachment index. Heat treatment of yeasts was performed at 56°C in PBS for 30 min, at 100°C for 10 min in a boiling water bath, or at 121°C for 10 min in the autoclave. Sodium periodate (10 or 50 mM; Sigma) oxidation of *Candida* carbohydrate moieties was performed as a pretreatment as described elsewhere (39). The effect of more acidic conditions on adherence was tested in citrate buffer at pH 4.5. Formalin killing of yeasts prior to attachment assays was achieved in a 4% (vol/vol) paraformaldehyde solution in PBS for 16 h at 4°C; this was followed by two PBS washing steps.

The following treatments were used in a competitive way (all reagents obtained from Sigma): the *Candida* acid protease inhibitor pepstatin A at concentrations of 0.1 to 100 μM, concanavalin A at 10 and 100 μg/ml, NHS (obtained from healthy volunteers) and heat-inactivated NHS (treated for 30 min at 56°C), heparin at 0.06 and 0.16 mg/ml, FCS, and bovine serum albumin at 0.5 mg/ml. Competition experiments were also performed with the following mono- and disaccharides (all used at a concentration of 2.5%, wt/vol, as described previously [27]): D-(+)-glucosamine, D-(+)-galactosamine, α-lactose, α-D-(+)-fucose, D-(+)-mannose, *myo*-inositol, D-sorbitol, D-(+)-galactose, D-(+)-glucose, DL-arabinose, and maltose.

For competitive protein and peptide inhibition assays, yeasts were added to each well along with different concentrations of each of the following extracellular matrix (ECM) proteins and the derived synthetic peptides (all obtained from Sigma except collagen type III and PepTite-2000, which were from Biomol-Telios Corp., Hamburg, Germany): human fibronectin (from human foreskin fibroblasts), RGDS, RGD, PepTite-2000, mouse laminin (purified from the Engelbreth-Holm Swarm mouse sarcoma), CDPGY-IGSR-NH_2, YIGSR, and human collagen type III.

Sodium azide (Sigma) was used at concentrations of 0.1 to 50 mM. Glutaraldehyde treatment of keratinocyte monolayers was performed as described before (42). Stimulation with gamma interferon (IFN-γ) to enhance intercellular adhesion molecule-1 (ICAM-1) expression on keratinocytes was done for 24 h at a cytokine concentration of 100 U/ml, as described previously (8). Expression of ICAM-1 by keratinocytes treated in that way was verified by immunocytochemistry (8).

Statistical methods. Statistical analysis was performed on all data by using Student's *t* test. For determination of the statistical significance of the differences between paired values of attachment, a 97.5% confidence level was chosen.

RESULTS

Effect of inoculum size and temperature on *Candida* adherence to cultured human keratinocytes. The binding of all wild-type *C. albicans* isolates tested was dependent on the inoculum size over the chosen concentration range of 0.75×10^3 to 5×10^5 yeast cells per well (Fig. 1). The attachment index increased in a nonsaturable fashion with higher concentrations of *C. albicans* inoculated. It was very interesting to note that upon the addition of high *C. albicans* inocula ($>10^5$) yeast cells tended to aggregate to a higher extent (>20 yeasts per aggregate) and displayed a less random distribution on the keratinocyte monolayer, as was observed with smaller inoculum sizes (Fig. 1). In parallel, more than 95% of all *Candida* cells displayed germ tubes after a 60-min exposure to keratinocytes in adherence assays at inocula of $\leq 10^5$ yeasts, whereas less than 20% (2.5×10^5 yeasts) or no germination at all (5×10^5 yeasts) was observed at higher yeast inocula (Fig. 1). When *C. albicans* adherence to cultured keratinocytes was tested at various temperatures, the highest attachment was observed at an incubation temperature of 37°C, while adherence at 4°C amounted to only approximately 20% of that seen at 37°C (Fig. 2). Similar results were obtained when Lee's medium instead of YNB or YNB at 37°C was used for growing liquid yeast cultures or when the time of *Candida* culture was varied between 16 and 48 h (data not shown). The potential influence of the carbohydrate source in the yeast growth medium (low and high glucose or galactose concentrations) on *C. albicans* adherence was also tested. Only the presence of 500 mM galactose in YNB led to a significant increase in *C. albicans* adherence; however, a higher tendency for coaggregation was

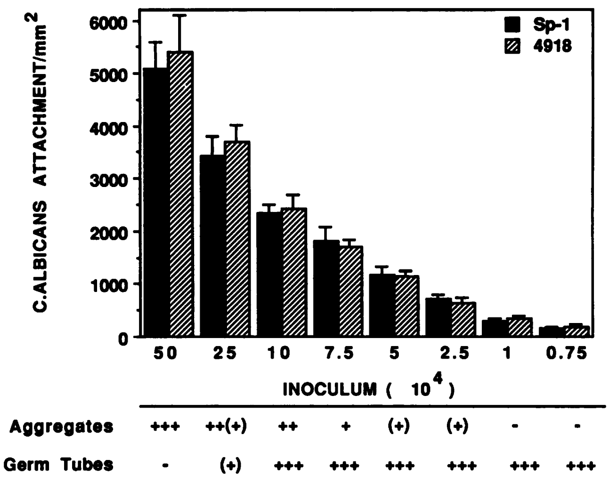

FIG. 1. Binding of *C. albicans* 4918 and Sp-1 to cultured human keratinocyte monolayers as a function of yeast inoculum size. The amount of yeast aggregation [−, no aggregation; (+), 1 to 5 yeasts per aggregate; +, 6 to 10 yeasts per aggregate; ++, 11 to 20 yeasts per aggregate; +++, >20 yeasts per aggregate] and the degree of germ tube formation [−, no germination; (+), 10 to 15% germination; +++, >90% germination] at each inoculum size are indicated at the bottom. Data represent means ± standard errors of four independent experiments.

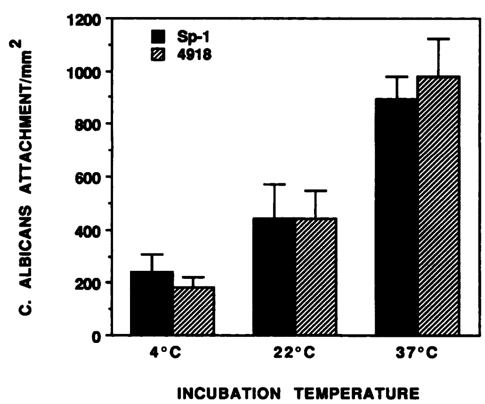

FIG. 2. *C. albicans* 4918 and Sp-1 attachment to cultured human keratinocyte monolayers as a function of incubation temperature. Data represent means ± standard errors of four independent experiments.

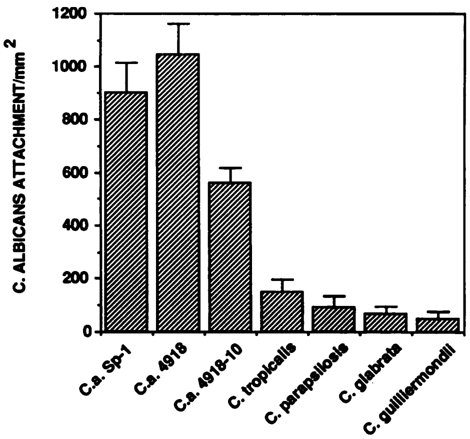

FIG. 3. Adherence of different *Candida* species to cultured human keratinocyte monolayers. Data represent means ± standard errors of four independent experiments. C.a., *C. albicans*.

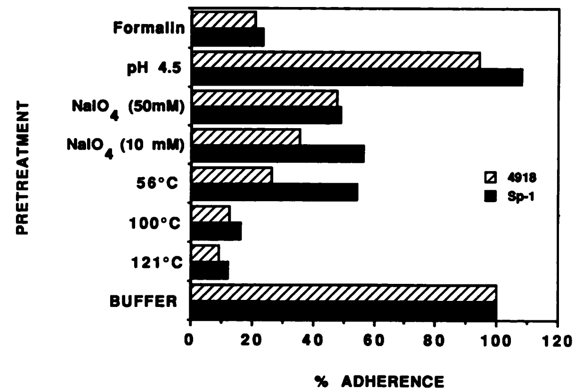

FIG. 4. Influence of various physical and chemical pretreatments on the binding of *C. albicans* 4918 and Sp-1 to cultured human keratinocyte monolayers. Data are indicated as relative adherence compared with a buffer control. Shown is one representative experiment out of four.

detected under these conditions. When germination of *C. albicans* was induced for 60 min in DMEM prior to adherence assays, we also observed higher attachment, with significantly higher *Candida* clumping (data not shown).

Adherence of various *Candida* spp. to keratinocytes. All of the *C. albicans* wild-type strains tested showed significantly higher attachment than other *Candida* spp. (Fig. 3). An avirulent mutant of *C. albicans* (4918-10) which is known to exhibit reduced adherence to other cells and tissues (34) was also reduced in its adherence to cultured human keratinocytes in comparison to its parent wild type ($P < 0.001$) (Fig. 3). The relative adherence of the various other *Candida* spp. was as follows: *C. albicans* > *C. tropicalis* ($P < 0.001$) < *C. parapsilosis* ($P < 0.001$) > *C. glabrata* ($P < 0.001$) > *C. guilliermondii* ($P < 0.001$). Of the other *Candida* species tested, none displayed more than a maximum of 17% adherence compared with the least adherent strain of *C. albicans*. This hierarchy of adherence was confirmed by using six additional *C. albicans* clinical isolates and two of each of the other *Candida* spp.

Influence of chemical and physical treatments of *C. albicans* on adherence. Heat treatment of yeasts at various temperatures significantly reduced *C. albicans* adherence to keratinocytes, which suggests the existence of heat-labile structures that are responsible for the attachment of *C. albicans* (Fig. 4). The most pronounced effects were seen after treatment at 100°C and autoclaving of the yeasts (121°C). Adherence was reduced to a lesser extent, but was statistically significant, by heat treatment at 56°C ($P < 0.001$). *C. albicans* adhesion was also effectively inhibited by formalin treatment of yeast cells prior to adherence assays ($P < 0.001$) (Fig. 4). Sodium periodate oxidation of sugar moieties on intact yeasts reduced *Candida* adherence by approximately 50% (Fig. 4). Acidic incubation conditions (pH 4.5) had no diminishing effects on *Candida* adherence (Fig. 4).

Effects of various potential inhibitors on *C. albicans* adherence. Active and heat-inactivated NHS both reduced *C. albicans* adherence to cultured keratinocytes to almost the same extent, thus suggesting the presence in serum of inhibitory agents that are not dependent on complement activation (Table 1). The mucopolysaccharide heparin reduced *Candida* adherence by approximately 50% (Table 1). Pretreatment of *C. albicans* with trypsin reduced adherence by 50% (Table 1). Pepstatin A, an inhibitor of the *C. albicans* secretory protease, was very effective in inhibiting adherence at concentrations as low as 1 μM (45% inhibition) (Fig. 5) (Table 1).

To determine whether energy-dependent processes are involved in *C. albicans* adherence to human keratinocytes, we used sodium azide. As can be seen in Fig. 6, sodium azide inhibited *C. albicans* adherence in a dose-dependent way by up to 80%. At least a 50% reduction in adherence was observed at a sodium azide concentration of 1 mM ($P < 0.005$). Preincubation of *C. albicans* at a concentration of 10 mM, however, had no significant inhibitory effect with Sp-1, while 4918 attachment was reduced by a margin of approximately 30 to 35% in several independent experiments ($P < 0.025$) (Fig. 6). To obtain further evidence that energy was mainly required on the host cell side for attachment to occur, we treated keratinocyte monolayers with glutaraldehyde prior to adherence assays. Under these conditions, the presence of 10 mM sodium azide in adherence assays led to a 17% reduction ($P < 0.025$) of *C. albicans* 4918 attachment, while *C. albicans* Sp-1 adhesion was not significantly affected (data not presented).

A significant enhancement of *C. albicans* adherence to human keratinocytes was seen with concanavalin A (Table 1). Concanavalin A preincubation of either *Candida* spp. or the keratinocytes led to similar high values, thus precluding *Candida-Candida* aggregation as the cause for the observed increase in attachment. *C. albicans* attachment was not affected by pretreatment of keratinocytes with IFN-γ, thus virtually ruling out an involvement of ICAM-1 as an adhesion receptor for *C. albicans* on human keratinocytes. No effect on adherence was seen with FCS and bovine serum albumin as competitive inhibitors (Table 1).

Protein-protein interactions in the attachment of *C. albicans* to human keratinocytes. To test whether Arg-Gly-Asp (RGD)-containing protein structures are of importance for *C. albicans* adherence to keratinocytes, we used various proteins containing the RGD sequence and relevant RGD peptides as specific inhibitors in adherence assays. Fibronectin showed a partial inhibition of *Candida* adherence with a reduction of slightly more than 30% at 250 μg/ml ($P < 0.005$) (Fig. 7). The synthetic RGD peptides RGDS and RGD (data not shown), which are integral parts of fibronectin, had no inhibitory effect on *Candida* attachment with the exception of PepTite-2000, which led to a 25% reduction ($P < 0.005$) in

TABLE 1. Effect of various agents on *C. albicans* 4918 adherence to cultured human keratinocytes

Treatment	Relative adherence (%) ± SE[a]
Buffer (DPBS)[b]	100
Pepstatin A	
0.1 μmol	92 ± 6
1 μmol	55 ± 5
Concanavalin A	
10 μg/ml	137 ± 10
100 μg/ml	210 ± 8
NHS	
Undiluted	45 ± 7
Diluted 1:10	72 ± 9
Heat-inactivated NHS	
Undiluted	55 ± 7
Diluted 1:10	80 ± 7
Trypsin	
0.5 mg/ml	76 ± 9
2.5 mg/ml	50 ± 7
Heparin	
0.06 mg/ml	71 ± 8
0.16 mg/ml	54 ± 9
FCS	
Diluted 1:5	99 ± 7
Bovine serum albumin	
0.5 mg/ml	98 ± 5
IFN-γ	
100 U/ml[c]	108 ± 10

[a] % Adherence = (adherence incubation mixture/adherence buffer) × 100. Data represent the means of three independent experiments.
[b] DPBS contains 137 mmol of NaCl, 2.7 mmol of KCl, 6.5 mmol of Na-phosphate, and 1.5 mmol of K-phosphate, pH 7.4.
[c] Cultivation of keratinocytes for 24 h prior to adherence assays in the presence of 100 U of IFN-γ per ml, as described before (8).

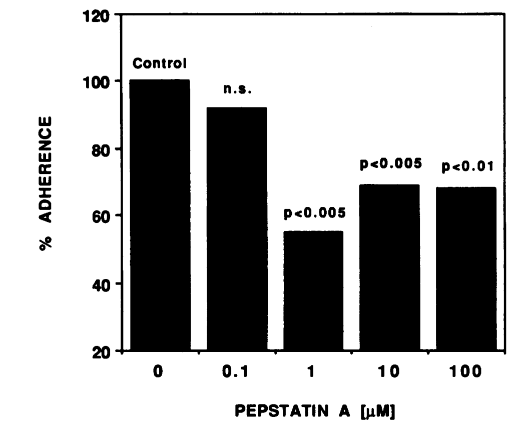

FIG. 5. Influence of the acid protease inhibitor pepstatin A on adherence of *C. albicans* 4918 to cultured human keratinocyte monolayers relative to a buffer control. Shown is one representative experiment out of four. n.s., not significant.

adherence (Fig. 7). The presence of laminin, on the other hand, was slightly inhibitory (Fig. 7). The use of the synthetic peptide CDPGYIGSR-NH_2, which is derived from the laminin B chain, led to a 76% ($P < 0.001$) reduction of *C. albicans* 4918 adherence to human keratinocytes and was by far the most potent inhibitor tested in this context (Fig. 7). However, the putative active sequence of this stretch, the pentapeptide YIGSR, alone was less effective ($P < 0.025$) (Fig. 7). With *C. albicans* Sp-1, both CDPGYIGSR-NH_2 and YIGSR showed similar activities and reduced adherence by a margin of approximately 20 to 25% (data not shown). Another ECM protein, collagen type III, was also a very effective inhibitor of *C. albicans* adherence in a dose-dependent fashion (Fig. 8).

Role of saccharide moieties in *Candida* adherence to human keratinocytes. Various sugars were tested for their potential to inhibit *Candida* adherence. Differential patterns of inhibitory activity were observed with the two *C. albicans* wild-type strains. A very potent overall competitive inhibition was found with the amino sugars glucosamine and galactosamine (Table 2). The inhibitory action of the amino sugars, however, was more pronounced with *C. albicans* 4918 than with Sp-1. The blocking activity of the other mono- and disaccharides also showed various degrees of inhibition, with α-D-(+)-fucose, α-lactose, and D-(+)-mannose being the most efficient inhibitors (Table 2). Adherence to keratinocytes of *C. albicans* Sp-1 was, in contrast to 4918, decreased much more effectively by α-D-(+)-fucose (53% inhibition), thus almost reaching the blocking activity of D-(+)-glucosamine (54% inhibition).

DISCUSSION

The present experimental model for testing *Candida* adherence to cultured human keratinocytes makes it possible to answer questions regarding the molecular adhesion events in the establishment of cutaneous candidosis. By using a well-defined and highly reproducible cell culture technique, our model system bears some advantages over other skin adherence assays that employ either exfoliated corneocytes in vitro or rodents in vivo (11, 35–37, 45). The principal advantage of using cultured cells for adherence assays lies in the potential to up- or down-regulate target cell surface proteins that might act as adhesion receptors for *C. albicans*

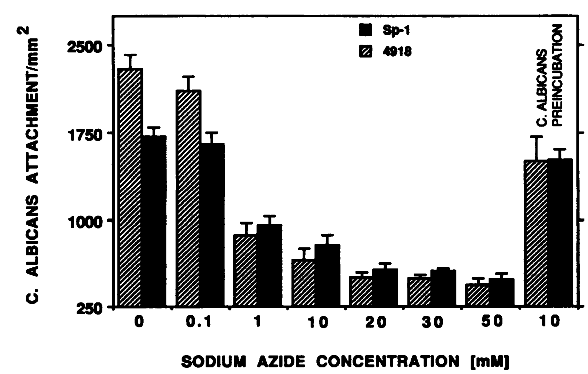

FIG. 6. Effect of the metabolic inhibitor sodium azide on *C. albicans* 4918 and Sp-1 adherence to cultured human keratinocyte monolayers. Azide was used either for coincubation (0.1 to 50 mM) in adherence assays or for yeast pretreatment (10 mM) with subsequent washings prior to yeast exposure to keratinocyte monolayers. Data represent means ± standard errors of four independent experiments.

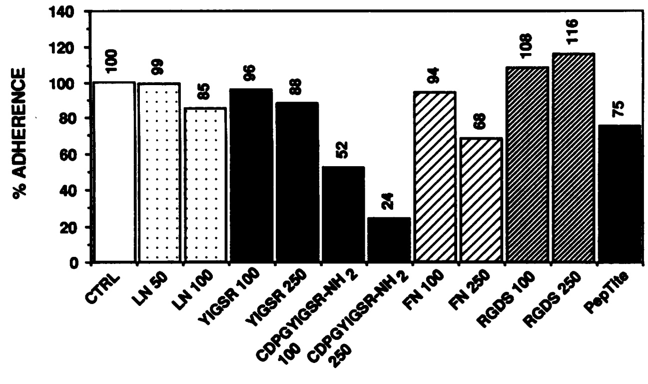

FIG. 7. Competitive inhibitory influence of various extracellular matrix proteins and derived synthetic peptides on the binding of *C. albicans* 4918 to cultured human keratinocyte monolayers. Laminin (LN) was used at 50 and 100 μg/ml; the synthetic laminin peptides YIGSR and CDPGYIGSR-NH_2 as well as the adhesive protein fibronectin (FN) and the derived peptide RGDS were all tested at 100 and 250 μg/ml. PepTite-2000 was used at 50 μg/ml. Data are indicated as the relative adherence compared with a buffer control (CTRL). Shown is one representative experiment out of three.

and, with regard to the present model, in modulating the degree of keratinocyte differentiation through a change in, e.g., Ca^{2+} and epidermal growth factor concentrations. The cells used in our approach expressed early markers of terminal differentiation but lacked a fully differentiated stratum corneum. Thus, the present epidermal cell culture model certainly relates to pathological conditions of cutaneous candidosis with defects present in the stratum corneum. Its relevance to the in vivo interaction of *Candida* spp. with corneocytes, however, has to be interpreted more cautiously because of the absence of a real stratum corneum in culture.

Our experiments with cultured human keratinocytes revealed that *C. albicans* was by far the most adherent of all *Candida* spp. tested (Fig. 3). The hierarchy of adherence was very similar to the findings of others in adhesion assays with cultured vascular endothelial cells (26, 39), intestinal cells (27), and exfoliated corneocytes and buccal epithelial cells (35). Adherence of *C. albicans* was dependent on the

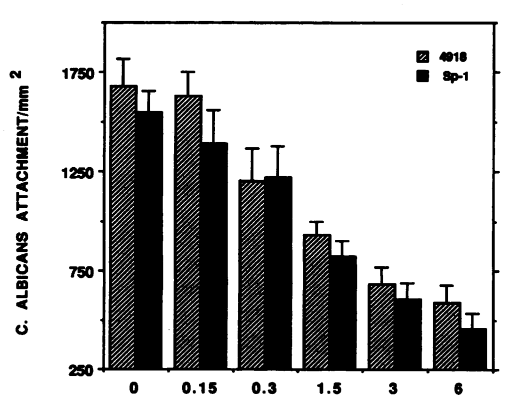

FIG. 8. Inhibitory activity of collagen type III on the adherence of *C. albicans* 4918 and Sp-1 to cultured human keratinocyte monolayers. Data represent means ± standard errors of four independent experiments.

TABLE 2. Effect of various sugar molecules on *C. albicans* 4918 and Sp-1 adherence to cultured human keratinocytes

Saccharide (2.5%, wt/vol)	Relative adherence (%) ± SE[a]	
	4918	Sp-1
Buffer (DPBS)[b]	100	100
D-(+)-Glucosamine	30 ± 4	46 ± 8
D-(+)-Galactosamine	57 ± 6	65 ± 4
α-Lactose	61 ± 11	54 ± 7
α-D-(+)-Fucose	62 ± 14	47 ± 2
D-(+)-Mannose	64 ± 14	52 ± 9
myo-Inositol	65 ± 7	68 ± 2
D-Sorbitol	78 ± 11	60 ± 1
D-(+)-Galactose	81 ± 2	71 ± 8
D-(+)-Glucose	83 ± 8	58 ± 3
DL-Arabinose	83 ± 8	58 ± 5
Maltose	98 ± 6	56 ± 4

[a] % Adherence = (adherence incubation mixture/adherence buffer) × 100. Data represent the means of three independent experiments.

[b] DPBS contains 137 mmol of NaCl, 2.7 mmol of KCl, 6.5 mmol of Na-phosphate, and 1.5 mmol of K-phosphate, pH 7.4.

inoculum size, with a nonsaturable binding behavior caused by yeast coaggregation (Fig. 1). This observation correlates very well with previous findings of adherence studies with a human intestinal cell line (27). At an inoculum of $\leq 10^5$ yeasts per well, germ tube formation of adherent *C. albicans* was uniformly observed (Fig. 1). Control experiments revealed that the germ tube induction was not caused by cell culture medium remnants or by the incubation buffer alone (data not presented). A close physical contact of *C. albicans* yeasts and keratinocytes seems to be the triggering event for germination. In other experiments aimed at determining the validity and comparability of the in vitro model, we found very good correlation to other *C. albicans* adherence models. For example, attachment of *C. albicans* was most pronounced at 37°C, which is in good agreement with previous results obtained with vaginal epithelial cells (25). These data may help to explain the clinical observation that cutaneous candidosis is most prevalent in skin regions with increased skin surface temperature due to occlusive conditions (32). Chemical and physical treatments of *C. albicans* yeasts had influences on attachment similar to that shown with human vaginal and buccal epithelial cells (44). Thus, heat- and formalin-killed yeasts were minimally adherent compared with viable *C. albicans* yeasts. As a summary of those basic experiments characterizing the model system, it can be delineated that *C. albicans* attachment to cultured human keratinocytes is guided by molecular events that are at least partly similar to those observed with other types of target tissue. In view of these findings, we wanted to better characterize the specific host-parasite relationships involved in the adherence mechanisms that initiate skin candidosis. To reach this goal, we used the potential inhibitory action of various agents in our in vitro test system.

In contrast to other parasites such as *Trypanosoma cruzi* which require parasitic energy for attachment to mammalian cells (42), *C. albicans* adherence seems to occur without major energy production on the fungal side, as can be seen from the inhibitory action of the metabolic inhibitor sodium azide (Fig. 6). The putative *C. albicans* adhesion receptors on the keratinocyte surface, however, seem to be down-regulated by the action of sodium azide. These findings lead us to suggest that energy-dependent processes are required for the expression of *C. albicans* adhesion receptors on the keratinocyte surface.

The very effective inhibition of *C. albicans* adherence by the acid protease inhibitor pepstatin A (Fig. 5; Table 1) underlines the potential importance of the *C. albicans* secretory protease in *C. albicans* adhesion events, as was already shown for attachment to oral epithelium (2), exfoliated corneocytes (11), and endothelial cells (12). However, by what molecular mechanism the secretory protease mediates adhesion remains an open question, since the pH during the adherence assay was maintained at 7.4, which seems to be in contrast to the reported low pH activity optimum of 3.5 to 4.5 of the *C. albicans* secretory protease (2, 32, 36). Therefore, others suggested that the action of the *C. albicans* secretory protease was limited to a zone of close physical contact between the yeast and the host cell, where a lower pH is reached in a microenvironmental gap (36). At present, however, we are unable to explain the inhibitory maximum of pepstatin A at 1 μM which we observed in several independent experiments (Fig. 5). A possible explanation for the decreased inhibitory activity at 10 and 100 μM could be the chemical nature of the pepstatin A molecule, which is characterized by rather hydrophobic side chains. Possibly, those residues could unspecifically enhance adhesion through bivalent interaction with the keratinocyte and the yeast cell surface at higher concentrations.

C. albicans is known to express a mammalian complement receptor type 3 (CR3; CD11b and CD18) analog which binds one of the physiological CR3 ligands, the human complement activation product iC3b, and mediates adherence to endothelial cells and to the ECM proteins fibronectin, collagen, and laminin in an integrin-like fashion (16, 21, 28, 34). If the *Candida* CR3 analog was to play an important role in adhesion to keratinocytes similar to what has been established for human endothelial cells or ECM, the interaction should be inhibitable by RGD-containing peptides, as proposed by several groups (10, 16, 28, 41). However, a role for an RGD-mediated adherence mechanism which has been clearly established for *C. albicans* adherence to endothelium is apparently of much less importance to the attachment to keratinocytes (Fig. 7). The synthetic peptides RGD and RGDS did not reveal any inhibition. Some inhibition, however, was observed with PepTite-2000, a specially designed RGD-containing peptide characterized by its great adhesive abilities. Fibronectin, which also contains an RGD sequence, had only a partial effect on *C. albicans* adherence (Fig. 7). Thus, our results correspond to the findings of Brassart et al. (5), who were not able to establish an RGD-mediated adhesion of *C. albicans* to human buccal epithelial cells. Therefore, RGD-mediated adhesion of *C. albicans* seems to be much more important in the attachment to endothelium than it is to epithelium in general. Further support for this notion is derived from experiments in which we tested whether *Candida* CR3 was potentially able to act as a counterreceptor to the physiological CR3 ligand ICAM-1 on the keratinocyte surface (8). IFN-γ-induced surface expression of ICAM-1 had no enhancing effect on *C. albicans* attachment to human keratinocytes (Table 1).

In search of other peptide sequences apart from RGD which mediate naturally occurring adhesive protein-protein interactions, we tested the peptide CDPGYIGSR-NH_2, which is part of the laminin B chain (15). This peptide was found to mediate tube formation of endothelial cells on laminin-coated surfaces (15). Furthermore, the sequence YIGSR has been shown to be the minimally required stretch for inhibition of metastasis formation of tumor cells in animal models (22). CDPGYIGSR-NH_2 was able to drastically reduce the adherence of *C. albicans* 4918 to cultured human keratinocytes (Fig. 7). A significant inhibition of adherence by CDPGYIGSR-NH_2 was also observed with *C. albicans* Sp-1, although it was less pronounced than that with 4918. Our findings are very interesting in the context of the results of Bouchara et al. (3), who demonstrated the existence of laminin binding proteins on *C. albicans*. Thus, it is intriguing to speculate that these proteins may act as mediators of adhesion to human keratinocytes. The possibility of such a molecular mechanism of *C. albicans* attachment to human keratinocytes is also supported by the fact that epithelial cells in general, and not terminally differentiated keratinocytes in particular, synthesize and deposit the ECM proteins laminin, fibronectin, and thrombospondin (18, 48).

It has already been shown by others that *C. albicans* is able to adhere to collagen-coated surfaces (28). Collagen III is a normal but minor constituent of adult skin (1). It was very effective in inhibiting *C. albicans* adherence to keratinocytes (Fig. 8). Type III collagen constitutes up to one-third of the total collagen of fetal and newborn skin and mucous membranes and is the predominant component of newly formed tissue (granulation tissue) in wound healing (1). It persists throughout life in decreasing proportions to type I collagen (1). Further studies are required to analyze the molecular importance of our findings, which should then provide answers to the question of whether collagen III acts primarily as an adhesion inhibitor on the yeast or on the host cell side. The clinical observation that *C. albicans* is frequently isolated from the collagen type III-expressing granulation tissue of lower leg ulcers (32) together with our blocking data, however, hint at the possibility that *C. albicans* might possess binding activity for collagen type III through a structure that is also important in *C. albicans* attachment to cultured human keratinocytes.

The use of mono- or disaccharides as inhibitors of *C. albicans* adherence to keratinocytes confirmed some of the data obtained previously with other cells and led us to the conclusion that lectin-type adhesion mechanisms are of importance in *Candida* attachment to cultured human keratinocytes (14). The patterns of inhibitory activity which we observed with two *C. albicans* wild-type strains suggest differential *C. albicans* attachment mechanisms that act with a lectin-type specificity, one being based mainly on the amino sugars glucosamine and galactosamine, as described for vaginal epithelial cells (43), and a second one which is much more susceptible to inhibition by fucose and mannose, as shown with buccal epithelial cells (40, 44) (Table 2). However, to get a more specific and detailed view of the role of carbohydrate-lectin interactions in the process of attachment of *C. albicans* to human keratinocytes, more complex oligosaccharide probes such as the Fucα1-2Galβ-bearing complex carbohydrates (5), the serotype A mannans (31), or the glycolipid structure lactosylceramide (23) need to be evaluated in the future for the keratinocyte adherence model.

In conclusion, as with other microorganisms such as streptococci (17), a complex set of multiple mechanisms leads to optimal adherence of *C. albicans* to cultured human keratinocytes. *Candida* attachment apparently involves a process of coaggregation, protein-protein interactions, and lectin-carbohydrate as well as mucopolysaccharide-based (heparin) interactions. The observed protein-protein interactions in the adherence of *C. albicans* to cultured human keratinocytes suggest molecular mechanisms distinct from those described for human endothelial cells (16) and more closely related to the mechanisms known for buccal epithelial cells (5). In addition to the apparent multiplicity of molecular adhesion mechanisms that exist in a single *C. albicans* isolate, there is also some variability in the expression patterns of those molecular mechanisms among different *C. albicans* strains.

The recent development of a spontaneously immortalized human keratinocyte cell line with strikingly similar cell surface characteristics compared with normal human keratinocytes makes possible the study of *Candida* adherence to keratinocytes in the future under even more standardized and reproducible conditions without the need for primary culture of human keratinocytes (4). Furthermore, if an even closer resemblance to the in vivo situation is required, *C. albicans* adherence to keratinocytes can also be tested in a new in vitro skin equivalent model consisting of both a dermal and a fully differentiated epidermal compartment (9).

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